

**Molecular identification and characterization of haptoglobin in teleosts
revealed an important role on fish viral infections**

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Abstract

Haptoglobin (Hp) molecule has been cloned and characterized in two marine teleosts (gilthead seabream and European sea bass), obtaining putative proteins of 319 residues encoded by an ORF of 960 bp in both species. However, the matrix of similarity revealed low identities among bony fish species 78.9% (seabream-sea bass), 43% (seabream/seabass-zebrafish) and lower than 20% with sharks and human. The protein sequences showed a signal peptide from the position 1 to 23, a trypsin domain from 47 to 297, and several predicted disulfide bridges and glycosylation sites. The expression of *hp* transcript levels during ontogeny showed a progressive increase of expression in seabream whilst remained almost unaltered in sea bass. By tissues, this gene was found constitutively expressed with the highest levels on liver in both species. The main results on *hp* transcript levels showed the up-regulation in gilthead seabream suffering from naturally occurring lymphocystis disease; and the down-regulation and up-regulation after nodavirus infection in the resistant gilthead seabream and the susceptible European sea bass, respectively. These findings demonstrate for the first time an important role of haptoglobin against viral infections, operating differently in two of the most important marine farmed fish species.

Keywords: haptoglobin; nodavirus; LCDV; teleost; gilthead seabream; European sea bass

Introduction

The animal reactions against infection, inflammation, or trauma are collectively known as the acute-phase response and comprise a wide range of pathophysiological responses to maintain the general homeostasis (Jain et al., 2011). Among these responses, liver produces a large number of plasma proteins which are known collectively as the acute-phase proteins (APP) being the most studied C-reactive protein (CRP) and serum amyloid P (SAP) and A (SAA). Acute phase response leads to increase some humoral components of the innate arm such as both complement and clotting systems, opsonins, antimicrobial peptides, lectins, lysozymes or metal-binding proteins among others. However, the contribution of APPs to fish immune response is not very well characterized (Bayne and Gerwick, 2001).

Haptoglobin (Hp) is the major hemoglobin-binding protein in the plasma of many vertebrates and all the mammals (Jayle et al., 1952; Wicher and Fries, 2006). Thus, this molecule binds to hemoglobin (Hb) forming Hp-Hb complexes and leading to the inactivation and clearance of oxidant radicals, and therefore it has a clear role in the oxidative stress (Alayash, 2011). Apart from being involved in the redox balance, Hp is considered a positive acute phase protein (APP), which has been reported to regulate the host immunity in mammals (Huntoon et al., 2008). In mammals, Hp has been reported to support the proliferation and functional differentiation of B and T lymphocytes (Huntoon et al., 2008) and affecting to the imbalance of T helper cell type 1/cell type 2 (Arredouani et al., 2003). Several APPs, including Hp as one of the most important, have been reported in different mammalian viral infections (Gómez-Laguna et al., 2010; Pomorska-Mól et al., 2012; Saco et al., 2016; Stenfeldt et al., 2011), and also in combination with bacterial infections (Pomorska-Mól et al., 2013), but most of them have only evaluated its levels in serum.

Among the most relevant virus of fish, lymphocystis disease virus (LCDV) is the causative agent of the main viral disease in cultured gilthead seabream, characterized by hypertrophy of fibroblastic cells in the connective tissue of skin (Borrego et al., 2015; Hick et al., 2016). On the other hand, nodavirus or nervous necrosis virus (NNV) produces viral encephalopathy and retinopathy altering the brain and retina structure and function (Breuil et al., 1991; Chaves-Pozo et al., 2012; Munday et al., 2002). NNV is nowadays considered one of the most serious viral diseases in marine aquaculture, which can be transmitted to the progeny through the gonad causing great mortality at early fish stages, especially in European sea (Breuil et al., 1991; Chaves-Pozo et al., 2012; Munday et al., 2002; Valero et al., 2015a).

In teleosts, some studies have evaluated the general role of APPs (including Hp) in different bacterial infections (Jayasinghe et al., 2015; Lin et al., 2007). However, little is known about the role of Hp in fish viral infections. Only one previous general study at protein level revealed the modulation of Hp levels in plasma by several agents such as yeast, bacteria and virus in rainbow trout (Gerwick et al., 2002) but nothing is known about its transcriptional regulation under naïve or infection conditions. To enlighten more about the fish *hp* gene and its role on antiviral immunity, the aim of this work was to clone and analyze its structure and evaluate its expression profile during the ontogeny, the tissue distribution and viral infections in gilthead seabream and European sea bass, two key farmed fish species in the marine aquaculture.

2. Material and methods

2.1 Animal maintenance and tissue

Adult specimens of the seawater teleost gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (100- 200 g and 300-500 g body weight, respectively) were breed at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia) and transferred to the University of Murcia aquaria. Fish were kept in 450-500 L running seawater (28 ‰ salinity) aquaria at $24 \pm 2^{\circ}\text{C}$ and with a 12 h light:12 h dark photoperiod. Animals were fed daily with 1 % body weight of a commercial pellet diet (Skretting) and acclimatized for 15 days prior to the experiments. These fishes were used for the *in vivo* infections as well as for tissue [brain, gill, gut, skin, liver, head-kidney (HK), spleen, thymus, gonad and blood] isolation by placing a piece of tissue in TRIzol reagent (Life Technologies) for later RNA isolation.

To obtain larvae, gilthead seabream and European sea bass healthy broodstocks were kept with natural seawater (38 ‰ salinity) and natural photoperiod at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia). Gilthead seabream and European sea bass larvae eggs were incubated at natural seawater (38 ‰ salinity) heated at $17 \pm 1^{\circ}\text{C}$ and filtered through mechanical and biological substrates. The temperature increased naturally and reached 26°C by the end of the developmental period studied. Gilthead seabream larvae were bred using phytoplankton with the “green water” technique (Papandroulakis et al., 2001) in a 5,000 L round tank with an initial density of about 60 eggs l^{-1} . During the experiment, the light intensity was 1,000 lux at the water surface, and the photoperiod was 16:8 (L:D). Water renewal was limited to 2 % daily during the first 20 days of culture and was achieved by the addition of 70 ml m^{-3} of a microalgae concentrated solution (Phytobloom, Necton) containing 80 % *Nanochloropsis oculata*. Subsequently, continuous water renewal (30 % h^{-1}) and light aeration were provided in the tank. Larvae were successively fed with enriched (Selco, Inve Animal Health) rotifers from 6 to 24 days post-hatching (dph), *Artemia* nauplii

(Inve Animal Health) from 20 to 35 dph, enriched Instar II *Artemia* from 31 to 58 dph and a commercial dry pellet diet (Skretting) from 54 dph onward. The sea bass larvae were kept in dark during the first 40 days upon hatching. Afterwards, the light intensity was 1,000 lux at the water surface, and the photoperiod was 16:8 (L:D). The specimens were subsequently fed with enriched Instar II *Artemia* and a commercial dry pellet diet (Skretting) from 54 dph onwards. Eggs and larvae at different time points from hatching (0, 3, 10, 13, 17, 24, 31 and 59 dph) were sampled and stored at -80°C in TRIzol reagent for later RNA isolation. The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) and the Bioethical Committee of the University of Murcia (Spain) and the Bioethical Committee of IEO (reference REGA ES300261040017) for the use of laboratory animals.

2.2 SSN-1, SAF-1 and DLB-1 cell lines

Cell lines were cultured at 25°C in 25 cm² plastic tissue culture flasks (Nunc) and maintained at exponential growth. The established striped snakehead SSN-1 (Frerichs et al., 1996), SAF-1 (Béjar et al., 2005) and DLB-1 (Morcillo et al., 2017) cell lines were cultured using Leibovitz's L-15-medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies), 100 µg ml⁻¹ streptomycin (Life Technologies), 100 U ml⁻¹ penicillin (Life Technologies) and 10 mM HEPES (Life Technologies). The medium osmolarity of DLB-1 was adjusted according to the sea bass serum (353 ± 2 mOsm kg⁻¹).

2.3 Bacterial infection

The bacterial strain *Vibrio anguillarum* R-82 was grown in tryptic soy broth (TSB) culture medium overnight at 22°C and continuous shaking. The culture was washed twice in phosphate-buffered saline (PBS), the optical density measured at 600 nm and bacterial

density adjusted to 10^8 bacteria ml^{-1} in PBS. They were then heat-killed at 60°C for 30 min, washed and stored at -80°C until used. Gilthead seabream specimens were divided into two groups, receiving an intraperitoneal (ip) injection of 1 ml of PBS alone (control group) or the same volume of PBS containing 10^8 bacteria ml^{-1} (infected group). Liver, HK and spleen tissues as well as peritoneal exudate leucocytes (PEL) were sampled after 4 h and 72 h of ip injection. Firstly, to obtain PELs 5 ml of culture medium were ip injected and the exudate collected by a slight peritoneal incision and a Pasteur pipette after 5 min of fish peritoneal massage. PELs were then washed by centrifugation and stored in TRIzol reagent at -80°C for later RNA extraction. Tissues were obtained by dissection and immediately stored in TRIzol reagent.

2.4 Natural LCDV infection

Specimens of gilthead seabream (average weight 41.6 ± 3.6 g) with clear symptoms of LCDV (white nodules, individually or in clusters distributed by the body surface) or apparently healthy (control) were kept in re-circulating aquaria with a flow water of 900 L h^{-1} , 28 ‰ salinity, $20 \pm 2^\circ\text{C}$ temperature and a photoperiod of 12h light: 12h dark. Control and diseased fish were sampled as described in a previous work (Cordero et al., 2016a). Skin and HK were dissected and immediately stored in TRIzol at -80°C for later RNA isolation.

2.5 NVV infection

For the *in vitro* assays, duplicate cultures of SAF-1 and DLB-1 cells were incubated for 24 h with culture medium alone (controls) or containing $50 \mu\text{g ml}^{-1}$ polyinosinic acid (pI:C; Sigma-Aldrich) or $10^6 \text{ TCID}_{50} \text{ ml}^{-1}$ NNV (strain 411/96, genotype RGNNV). After treatment, monolayers were carefully washed with PBS and stored in TRIzol reagent (Life Technologies) at -80°C for later RNA isolation.

For the *in vivo* trial, specimens of gilthead seabream or European sea bass specimens received a single intramuscular injection of 100 µl of SSN-1 culture medium (mock-infected) or culture medium containing 10^6 NNV TCID₅₀ (Chaves-Pozo et al., 2012) since this route of infection has been proven as the most effective (Aranguren et al., 2002). Both species were sampled 1, 7, 15 days after the viral injection and brain, HK and gonad were stored for RNA isolation. Whilst seabream is reservoir and resistant (no mortality) to NNV infection, sea bass was susceptible with a mortality of 55% (Chaves-Pozo et al., 2012).

2.6 RNA extraction

Samples from the above experiments were homogenized with a pellet pestle and total RNA was extracted from each tissue with Trizol reagent (Life Technologies) according to the manufacturer's instructions. RNA present in samples was then quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. The RNA was treated with DNase I (Promega) to remove genomic DNA contamination. To check the RNA quality, an agarose gel was also run. Complementary DNA (cDNA) was synthesized from 1-2 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer.

2.7 Molecular cloning of *haptoglobin*

According to published sequences of *hp* orthologous we performed a BLAST analysis through NCBI database (<http://www.ncbi.nlm.nih.gov/>) against the gilthead seabream and European sea bass expressed sequenced tags (EST). When identified, mRNA sequence was obtained according to the 3' RACE and 5' RACE protocols (Life Technologies) for the 3'-UTR and 5'-UTR amplification.

Samples were run by nested PCR with the corresponding primers in each case (Table 1). Amplification was performed in 20 µl samples containing 2.5 µl of PCR reaction buffer

(10×, Life Technologies), 2 µl forward and reverse primers for *hp* in gilthead seabream and European sea bass (10 mM each), 0.5 µl dNTP mix (2.5 mM each), 1.25 µl MgCl₂ (50 mM), 0.25 µl Taq DNA polymerase (5 U l⁻¹, Life Technologies), 15.25 µl DNase/RNase-free distilled water (Life Technologies) and 2 µl cDNA from the liver. PCR reactions were performed in a MasterCycler Gradient PCR: 94°C for 5 min, 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and followed by 72°C for 10 min. The PCR products were separated in a 1.2% agarose (Pronadisa) gel containing 0.01% (v/v) of Red Safe[®] (Life Technologies) and visualized under UV light. Bands were picked up and DNA extracted with GenJET Gel Extraction Kit (Thermo Scientific) according with the manufacturer's instructions. Ligation was performed with an incubation of 20 min containing 4 µl of eluted DNA, 1 µl of salt solution and 1 µl of pcDNA 3.1/V5-HIS TOPO vector (Invitrogen). Transformation was performed using TOP10 chemically competent cells (Life Technologies) according to the manufacturer's instructions. Competent cells were spread in Luria Agar (Pronadisa) plates and incubated at 37°C for 24h. Next day, a single colony was inoculated in Luria Broth (Pronadisa) with mixing at 250 rpm at 37°C for 24h. Then, the plasmid was isolated with Gen Elute[™] Plasmid Mini Kit I (Sigma-Aldrich). The DNA was quantified by Nanodrop[®] as described elsewhere. Concentration was adjusted to 100 ng µl⁻¹ to carry out the Sanger sequencing (Sanger et al., 1977).

2.8 Real-time PCR study

The expression of *hp* gene in both fish species was analyzed by real-time PCR (rtPCR), which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) as described elsewhere (Cordero et al., 2015) and using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). For each *hp* mRNA, gene expression was corrected by the elongation

factor 1 α (*ef1a*) content in each sample. Information of primers is listed in Table 1. In all cases, each PCR was performed twice to confirm the results.

2.9 Bioinformatic analysis

After sequencing process, files were primarily analysed by Chromas software version 2.4.4. Next, we found the ORF and encoded protein with DNASTAR Lasergene software version 7.1. The putative protein features were obtained with ProtParam tool (<http://web.expasy.org/protparam/>). A ClustalW was made with similar protein sequences after BLASTp analysis with different organisms in the evolution-scale, and then the Neighbor-joining tree was performed with MEGA software version 7.0 (Kumar et al., 2016). The alignments with the conserved domains were performed with BioEdit software version 7.2.5 (Hall, 1999). The signal peptide and the glycosylation sites were retrieved with SignalP 4.1 and NetNGlyc 1.0 servers, respectively.

2.10 Statistical analysis

The results are expressed as mean \pm SEM. Data of challenges were statistically analyzed by T-student test to determine differences between groups. Data of expression from tissues and fish ontogenetic profile were statistically analyzed by one-way ANOVA and Tukey's post-hoc test. All the statistical analysis were conducted using Statistical Package for Social Science (SPSS for Windows; v19.0, USA) and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 Sequence of *hp* in gilthead seabream and European sea bass

The results of cloning and sequencing the haptoglobin in both species revealed mRNAs with 5'-UTR of 38 and 32 bp and 3'-UTR of 178 and 174 bp for gilthead seabream and

European sea bass, respectively; with both open reading frames (ORF) of 960 bp, which encode a polypeptide of 319 amino acids in both cases. The predicted molecular weight (MW) and the theoretical isoelectric point (pI) was 34.9 kDa and 7.6 for gilthead seabream and 35.1 kDa and 8.5 for European sea bass. Both sequences were submitted to NCBI databank with the following accession numbers KU940258 for gilthead seabream and KU940259 for European sea bass.

3.2 Structural and phylogenetic comparative analysis of haptoglobin

After BLAST analysis, the alignment of Hp sequences with other from important teleosts as well as from cartilaginous fish and mammals, including human, revealed a high variability of this molecule (Figure 1). We found no alignment matches with birds, reptilians or lamprey species. In addition, we only found three conserved domains between both species (positions 70 to 85, 158 to 174 and 242 to 256). Globally, there are only 27 conserved residues across all the analyzed species (Figure 1). Despite both sequences have 319 aa, the similarity of gilthead seabream and European sea bass was 78.9% whilst zebrafish showed similarity of only 42.3% with both studied species, human showed a similarity of 18.6% and 19.7% with gilthead seabream and European sea bass, respectively, and surprisingly the similarity was even lower (15-18%) with cartilaginous fish (Table 2).

The Neighbor-joining phylogenetic tree was also constructed and reflected three different clusters, one cluster with all the teleost fishes (including our two species), other cluster with mammals (including mouse and human) and the farthest cluster with cartilaginous fishes (Figure 2A). Regarding the putative protein structure, signal peptide of 23 aa and trypsin domains (positions 49 to 247) were found in both species (Figure 2B). About the conserved cysteine residues, we have found two disulfide bridges (between 214-233 and 244-273) in gilthead seabream and three disulfide bridges (49-297, 214-233 and 244-273) in European

sea bass, whilst potential N-glycosylation sites were found in the residues 2, 141 and 302 for gilthead seabream and 96, 123, 141 and 302 for European sea bass. Interestingly, SUSHI domain is missing in all the teleost fishes analyzed, including gilthead seabream and European sea bass, while two were found in cartilaginous fishes and human but only one SUSHI domain in mouse (Figure 2B).

3.3 Haptoglobin is constitutively expressed in both gilthead seabream and European sea bass

During the ontogeny, the transcript profile of *hp* revealed very low expression at all the analyzed stages from eggs to 73 dph in both species (Figures 3A and 3B). In gilthead seabream, the expression of *hp* was increasing constantly to 59 dph reaching a significant level compared to the rest of larval stages (Figure 3A); whilst in European sea bass high variability in the *hp* transcript profile was found from eggs to 13 dph achieving stability from 17 dph onward (Figure 3B). Interestingly, *hp* transcription was around ten-fold higher in seabream than in sea bass.

In naïve adults, the transcript profile of *hp* in tissues (Figure 3C and 3D) revealed its constitutive expression in all the assayed tissues as well as in the gilthead seabream cell line SAF-1, and in the European sea bass cell line DLB-1. In both species, liver showed the highest levels of *hp* transcript. Strikingly, and compared to the transcription during development, *hp* gene expression was around ten-fold lower in seabream compared to sea bass tissues.

3.4 Role of *hp* on fish bacterial infections

Based on the idea that Hp is an acute phase protein involved in bacterial infections we did a preliminary test to ensure this. Thus, our data showed the up-regulation of *hp* transcription

in gilthead seabream liver, HK, spleen and peritoneal exudate leucocytes after 4 h of ip injection of *Vibrio anguillarum* (Supplementary Figure 1).

3.5 LCDV natural outbreak up-regulated the *hp* transcript level

During a natural outbreak of the LCDV in gilthead seabream we evaluated the transcript levels of *hp* in the main lymphomyeloid organ, the HK, as well as in the target tissue of LCDV, the skin. The gene expression levels of *hp* after a natural outbreak of LCDV was significantly up-regulated in HK whilst it showed no differences in the skin, both compared to these tissues in the control or non-infected group of gilthead seabream (Figure 4).

3.6 NNV infection down-regulates *hp* transcription in seabream but up-regulates it in sea bass

We evaluated the transcription of *hp* after *in vitro* or *in vivo* NNV infections in gilthead seabream and European sea bass. First, poly I:C-treated and NNV-infected seabream SAF-1 cell line showed down-regulated transcription of *hp* (Figure 5A) whilst it was unaffected in the case of the sea bass DLB-1 cell line (Figure 5E).

In *in vivo* NNV infections, the expression of *hp* was evaluated in the target tissue of NNV, the brain, the primary lymphomyeloid organ, the HK, and the gonad, which is used to vertically transmit the virus. In the case of seabream (Figure 5B-D), a resistant species to the NNV strain used, *hp* transcription was significantly down-regulated in the brain (Figure 5B) 1 day post infection and in the HK (Figure 5C) after 7 days of infection whilst no changes were observed in the gonad (Figure 5D). In the case of European sea bass (Figure 5F-H), a very susceptible species, *hp* transcription was up-regulated in the brain (Figure 5F) after 15 days of infection but interestingly it was significantly up-regulated in HK (Figure 5G) and gonad (Figure 5H) tissues at all the assayed times, reaching up to 1,000-fold increments in the gonad after 7 days of NNV infection.

4. Discussion

In general, haptoglobin has been mainly studied in relation with its capacity to bind hemoglobin (Alayash, 2011; Dobryszczyka, 1997; Polticelli et al., 2008; Wicher and Fries, 2006). In the last years, however, few studies have focused their interest on the role of haptoglobin in immunity (Huntoon et al., 2013, 2008).

From an evolutionary perspective, Hp appeared in fish but is not present in birds and amphibians (Wicher and Fries, 2006). Phylogenetic analysis with the available sequences of haptoglobin revealed strong differences among species. In general, the multiple alignments and the neighbor-joining tree matched for each species, but surprisingly, rainbow trout and Atlantic salmon showed very close genetic distance according to the neighbor-joining tree but quite different putative sequences according with the alignments of their sequences with 358 and 316 residues, respectively, which may indicate different evolution and/or function in these genetically similar species. On the other hand, in our two fish species, despite of showing lower similarity according with the neighbor-joining tree, the alignment of their sequences revealed that gilthead seabream and European sea bass are similar with 319 residues in both cases, suggesting a conserved function of this molecule in these two Mediterranean farmed fish species. Comparing the domains we found a very curious finding: cartilaginous fishes showed two SUSHI domains in a similar way than human, whilst mouse contains only one SUSHI domain, approaching the structure of human haptoglobin closer of cartilaginous fishes rather than mouse. The function of this protein deserves to be investigated as well as why these domains are not present in bony fish.

The acute phase proteins have been scarcely studied in fish (Bayne and Gerwick, 2001; Gerwick et al., 2002; Lin et al., 2007; Peatman et al., 2007). Among them, little attention has been paid to haptoglobin so far. At protein level, Hp has been only partially detected in plasma of rainbow trout (Gerwick et al., 2002), but not detected in 2D proteomic characterizations of fish serum (Brunt et al., 2008; Douxfils et al., 2011; Ghisaura et al., 2014; Isani et al., 2011; Russell et al., 2006) or mucus (Cordero et al., 2016c, 2015; Jurado et al., 2015; Patel and Brinchmann, 2017; Rajan et al., 2013, 2011; Sanahuja and Ibarz, 2015). More data are found at transcriptomic level where *hp* is easily found in skin (Valenzuela-Muñoz et al., 2017), liver (Asker et al., 2013), heart (Marques et al., 2008), larvae (Wang et al., 2014) and whole fish body (Meijer et al., 2005) of several fish species. In our study, we found that *hp* is constitutively expressed in all the analyzed tissues from gilthead seabream and European sea bass, with the highest transcript levels in liver. During the ontogenetic development, *hp* was expressed in all the larvae stages including eggs. However, the pattern of expression during ontogeny was very different in the two species; whilst the transcriptional levels of *hp* were concomitantly increasing over the time in gilthead seabream, we have observed a decrease of *hp* transcript levels around 13-17 dph in European sea bass. These transcriptional differences between gilthead seabream and European sea bass have been also observed in other molecules such as non-specific cytotoxic cell receptor protein (*nccrp1*) (Cordero et al., 2016b), suggesting an important role, and more remarkable in European sea bass, of *hp* in early larval stages.

Regarding the fish antibacterial activity, many papers have considered different molecules such as *g-lysozyme*, *leap-2* or *β -defensin* after bacterial infections (Buonocore et al., 2014; Cuesta et al., 2011; Li et al., 2015) but only one previous report focused on haptoglobin and showed an increase of Hp protein abundance after *Vibrio* infection in trout plasma

(Gerwick et al., 2002). These data are correlated with our transcriptional finding, in which the expression of *hp* was increased in liver, HK, spleen and peritoneal exudate leucocytes after *V. anguillarum* i.p. injection suggesting an important role against bacterial infections. All these data point to, and demonstrates, the importance of APPs, and concretely Hp, on the fish fight against bacteria.

However, the role of haptoglobin after viral infections has not been previously approached. At transcriptional level, a short communication reported serum amyloid A (*saa*) and *hp* modulation by poly I:C induction in liver of black rockfish (*Sebastes melanops*) (Jayasinghe et al., 2015). For this reason, we focused this study using two viral models: LCDV in gilthead seabream and NNV in seabream and European sea bass. In the case of LCDV, little is known about seabream immune response against this virus. Our previous study showed the up-regulation of *nccrpl* in HK but not in skin of naturally infected gilthead seabream (Cordero et al., 2016a), similarly to what happens in the present study for *hp* transcripts. In other fish species, after LCDV infection at larvae stages, molecules involved in cell division and apoptosis were down-regulated in fins as showed in a microarray assay in turbot (*Psetta maxima*) (Iwakiri et al., 2014). However, in agreement with our study, *grim19* was also up-regulated in the HK of infected turbot (Wang et al., 2014). All these data demonstrates an important induction of the immune response in LCDV-fish in the HK tissue but very low or inefficient induction in the target tissue, the skin, but anyhow the fish overcome the disease and recover. By contrast, antimicrobial peptides (AMPs) such as piscidin 3 are increased in the skin of naturally infected gilthead seabream (Dezfuli et al., 2012).

Regarding the infections with nodavirus, one of the most important marine virus, we found a great inhibition in the *hp* gene expression levels in the seabream SAF-1 cell line upon

infection and no effect on the European sea bass DLB-1 cell line, which is parallel to the effects observed for the poly I:C administration. These data could be due to the differential cell-type origin of the cell lines and probably different regulation. In *in vivo* infections, the study of the brain as the target tissue of NNV, the HK as the main hematopoietic organ, and the gonad that transmits the virus to the progeny, is basic to understand the viral disease. The expression levels of *hp* were much more inducible in European sea bass than in gilthead seabream after nodavirus infection. These data are in accordance with the transcript levels found in other immune-related molecules such as *tnfa*, *mx* or *nkef* after *in vivo* nodavirus infection in HK of European sea bass (Esteban et al., 2013; Poisa-Beiro et al., 2008; Valero et al., 2015b). Importantly, the greatest fold change in *hp* after infection was found in European sea bass gonad (more than 1,000-fold at 7 days), but in gilthead seabream *hp* was not induced or even down-regulated, which taken altogether with the inhibition of *hp* transcripts found in brain and HK of nodavirus-infected gilthead seabream may be in relation with the asymptomatic effects of nodavirus in this fish species. This hypothesis is supported by the absence of modulation of other molecules involved in inflammation such as *il1 β* due to a short but effective response against the virus in the brain (López-Muñoz et al., 2012).

Overall, we have performed the molecular identification and characterization of haptoglobin, a very relevant but also unknown acute phase proteins. The protein comparison and phylogenetic tree revealed that the structure of haptoglobin is highly variable among species. In our study, this molecule is constitutively and widely expressed from eggs to adults of gilthead seabream and European sea bass. In addition, the role of haptoglobin in viral infections was unravelled for first time in fish, being inducible in gilthead seabream under LCDV, but not under NNV infections, in both cases virus that do

not produce fish death. However, in European sea bass, NNV, which is able to kill the fish, up-regulated the transcription levels of this molecule, demonstrating differential induction of this acute phase molecule depending on the species and pathogens and enlighten new strategies to understand the viral immunity at molecular level.

Acknowledgements

Dr. H. Cordero is grateful to the Spanish Ministry of Economy and Competitiveness (MINECO) for a grant (BES-2012-052742). This work was co-funded by several national projects of the MINECO and MINECO-FEDER (grant numbers AGL2011-30381-C03-01, AGL2013-43588-P and AGL2014-51839-C5-1-R) and *Fundación Séneca de la Región de Murcia (Grupo de Excelencia* grant number 19883/GERM/15). The technical help in fish maintenance by Dr. M. Arizcun and IEO staff are acknowledged as well as to AI Salvá at the University of Murcia. Authors are grateful to Dr. J. Meseguer as emeritus research group leader. Authors appreciate Dr. C. Flores and the staff of Molecular Biology Service from University of Murcia for the technical support with Sanger sequencing. We also thank Dr. P. Morcillo and Dr. Y. Valero for providing samples from cell lines and NNV infections. Nodavirus and *Vibrio anguillarum* strains were kindly donated by Dr. P. Fernández-Somalo (*Laboratorio Central de Veterinaria*, Algete, Spain) and by Dr. A.E. Toranzo (University of Santiago de Compostela), respectively.

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Figure legends:

Figure 1. Multiple alignment of the predicted gilthead seabream and European sea bass haptoglobin amino acid sequences with other known haptoglobin molecules. The identical (*) and similar (. or :) residues are indicated. Accession numbers: gilthead seabream *Sparus aurata* (KU940258), European sea bass *Dicentrarchus labrax* (KU940259), rockfish *Sebastes schelegelii* (AKI07271), Nile tilapia *Oreochromis niloticus* (XP_003445796), Atlantic salmon *Salmo salar* (XP_014019196), rainbow trout *Oncorhynchus mykiss* (CDQ94979), channel catfish *Ictalurus punctatus* (AHH39487), pufferfish *Tetraodon nigroviridis* (CAF94447), zebrafish *Danio rerio* (XP_009301802), mouse *Mus musculus* (NP_059066), human *Homo sapiens* (AAI21126), nurse shark *Ginglymostoma cirratum* (AEB61473) and little skate *Leucoraja erinacea* (AFN85000).

Figure 2. A: Neighbor-joining tree of the available cartilaginous and bony fish, mouse and human propeptide haptoglobin sequences. 0.1 indicates the genetic distances, which were calculated based on protein differences (p-distance) with pairwise deletion of gaps. The number at each node indicates the percentage of bootstrapping after 1,000 replications. Accession numbers are specified in Fig. 1. B: Identification and organization of gilthead seabream and European sea bass putative haptoglobin protein domains with those of cartilaginous fish, mouse and human haptoglobin.

Figure 3. Transcript levels of *haptoglobin* during the ontogeny (from 0 to 60 days post-hatching) in gilthead seabream (A) and European sea bass (B). *Haptoglobin* expression profile in different adult naïve organs (brain, gill, gut, skin, liver, head-kidney, spleen, thymus, gonad, blood), and SAF-1 or DLB-1 cell lines from gilthead seabream (C) and

European sea bass (D), respectively. Results are expressed as the mean relative expression to the expression of endogenous control *efla* gene \pm SEM (n=3). Different letters denote significant differences among ontogeny stages or among organs/cell lines when $p \leq 0.05$.

Figure 4. *Haptoglobin* gene expression profile in the head-kidney and skin of non-infected (white bars) and LCDV-infected (black bars) gilthead seabream during a natural outbreak. . Results are expressed as the mean relative expression to the expression of endogenous control *efla* gene \pm SEM (n=5). Asterisks denote significant differences between control and infected groups when $p \leq 0.05$.

Figure 5. *Haptoglobin* transcription after nodavirus (NNV) infections (A-H). Relative gene expression of *haptoglobin* in SAF-1 (A) and DLB-1 (E) cell lines after poly I:C (grey bars) and NNV (black bars) treatments compared with controls (white bars). *Haptoglobin* profile after 1, 7 and 15 days of mock (white bars) or NNV infections (10^6 TCID₅₀ per fish; black bars) in the brain (B, F), head-kidney (C, G) and gonad (D, H) from gilthead seabream (A-D) and European sea bass (E-H), respectively. Results are expressed as the mean relative expression to the expression of endogenous control *efla* gene \pm SEM (n=3). Asterisks denote significant differences between control and poly I:C or NVV groups when $p \leq 0.05$.

Table 1. Primers used for rtPCR study.

Gene name	Symbol	Species	Acc. number	Sequences (5' → 3')	Use
<i>elongation factor 1 alpha</i>	<i>ef1a</i>	Gilthead	AF184170	F1: TGTCATCAAGGCTGTTGAGC	rtPCR
		seabream		R1: GCACACTTCTTGTTGCTGGA	
		European sea	FM019753	F1: CGTTGGCTTCAACATCAAGA	rtPCR
		bass		R1: GAAGTTGTCTGCTCCCTTGG	
<i>haptoglobin</i>	<i>hp</i>			F1: TTCCTCTTACTTGCCCTGGA	rtPCR
		Gilthead	KU940258	R1: CAGGGCCTGAAGCTCTACTG	rtPCR
		seabream		R2: AGAGCGTTTCATCCTTTCTT	5' RACE
				R3: CATAACCACAGCCTTTTCA	5' RACE
				F1: CTTTGACTGTGCTCCTGCTG	rtPCR
		European sea	KU940258	R1: ACGGAGTGATGCTACCTTGG	rtPCR
		bass		F2: TTGTTCTCCATCCAGGCTTC	5' RACE
				R2: GATGGGGGTCACTTCATCAC	5' RACE

Table 2. Identity matrix (%) with gilthead seabream and European sea bass haptoglobin.

Species	Accession number	Gilthead seabream	European sea bass
Gilthead seabream (<i>Sparus aurata</i>)	KU940258	100	78.9
European sea bass (<i>Dicentrarchus labrax</i>)	KU940259	78.9	100
Rockfish (<i>Sebastes schelegelii</i>)	AKI07271	71.4	74.2
Nile tilapia (<i>Oreochromis niloticus</i>)	XP_003445796	70.5	74.6
Atlantic salmon (<i>Salmo salar</i>)	XP_014019196	60.8	63.9
Rainbow trout (<i>Oncorhynchus mykiss</i>)	CDQ94979	53.9	58.3
Pufferfish (<i>Tetraodon nigroviridis</i>)	CAF94447	53.0	57.5
Channel catfish (<i>Ictalurus punctatus</i>)	AHH39487	49.0	51.2
Zebrafish (<i>Danio rerio</i>)	XP_009301802	42.3	42.3
Mouse (<i>Mus musculus</i>)	NP_059066	19.9	20.6
Human (<i>Homo sapiens</i>)	AAI21126	18.6	19.7
Nurse shark (<i>Ginglymostoma cirratum</i>)	AEB61473	17.6	18.7
Little skate (<i>Leucoraja erinacea</i>)	AFN85000	15.1	14.9

Figure 1

Gilthead seabream	-----	1
European sea bass	-----	1
Rockfish	-----	1
Nile tilapia	-----	1
Atlantic salmon	-----	1
Rainbow trout	-----	1
Channel catfish	-----	1
Pufferfish	-----	1
Zebrafish	-----MRRKFILTNPAVLTPFAAAGGR	23
Mouse	MRALGAVVTLTLLWGQLFAVELGNDAMDDEDSCPKPEIANGYVEHL-----VRYRCRQ-FYRLRAEGDGVYTL	68
Human	MSALGAVIALLLWGQLFAVDGNDVTDIADGCKPKPEIAHGYVEHS-----VRYQCKN-YKRLRTEDGQVYTL	68
Nurse shark	-MGSLSLQLVLLCTLLGCTLQDHVETDHSKVGCPVFNITHGHYELTHHDEDTYLTIVRYVCDAPVYHLEHPEDATFVC	79
Little skate	-MWFLVLNLALSSVL--LADH-----HIDCEPTNITHGHFHTITAEGHHAIGSVIKYECNSPRWFEDEHDIYVC	70
Gilthead seabream	-----MDITSKMWFSLTVLLLAT--WAGL	22
European sea bass	-----MNIITKRMWFSLTVLLAA--WAGL	22
Rockfish	-----MNIINNMWFSLTVLLAA--LACL	22
Nile tilapia	-----MNIIMHMAFSLTVVVLLA--WAGL	22
Atlantic salmon	-----MWSELVLLAASCVCLEQD	19
Rainbow trout	-----MGGQTGGESFLQFWSYNFSRSTSSQLRRHSSTYKRLINRYFHONVPIPGSAPRGLLCT	57
Channel catfish	-----MKWESVAVFLVGL--MIGL	17
Pufferfish	KKEKEKRRRRKRLGLEAGLVTSLAQALFLQSFKSPYRYKYQSFVKLTLPKEPKPSALKDKMWFSLTVLLAA--WAGL	100
Zebrafish	VQRVEVLVDWLQCCRPVQPSFMPLTGTYYKAEQFKSMRLVLLSSGNHNFVMEQLFLVIFRLSVAVLLGT--LTL	100
Mouse	ND-----EKQVMT	77
Human	NDKKQWINKAVGDKLPECEADDGCKPKPEIAHGYVEHSVRYQCKNY-----KLRTEDGQVYTLNNEKQWINK	136
Nurse shark	TDDNGWTNAKLKHLPECHKVA-CGKPPAVDGHGFYITTFGVDNYLSAVKYTCDDNYHERDFSEGYVCTIDGKWRNT	158
Little skate	ASNGHWKNKARAEVLVPCQLIH-CHAPHHIENATLEYLTSRHDVYHSIIQYHCIDDHIEEKYSDEGIYVCAISGHWENK	149
Gilthead seabream	ADVHAHEERMRKRSVSASRSASCRSRRMIGGNSA--PHVPWQAVVYLSDEVLLGGYAGGALISDRWVLTAGRN-LFVVKSR	99
European sea bass	ADVPHTEERMRKRSVSASKVASLRSRRMIGGTLA--PHVPWQAVVYHSDVLLGGYAGGALISDRWVLTAGRN-VFVVKSR	99
Rockfish	ADV--TEERVKRSVSASGLASLRSRRMIGGTLA--PHVPWQAVVYHSDVLLGGYAGGALISDRWVLTAGRN-VFVVKSR	96
Nile tilapia	ADVHAHEERMRKSVLASRTASLRSRRMIGGTLA--PHVPWQAVVYTAESILLGGYAGGALISDRWVLTAGRN-LFVVKSR	99
Atlantic salmon	KIKIKIKIKIKSIDEMSDNLSLRSRRMIGGTLA--PHVPWQAVVYLSKNVMGGFAGGALISDRWVLTAGRN-LFVVKSR	96
Rainbow trout	KDKIKIKIKIKSIDEIPNNSDLRSRRMIGGTLA--PHVPWQAVVYLSKNVMGGFAGGALISDRWVLTAGRN-LFVVKSR	134
Channel catfish	SESR-VVDGKHEATEANTDALRSRRMIGGTLIP-GHVPWQAVVYLSKLLGGYAGGALISDRWVLTAGRN-LFVVKSR	94
Pufferfish	ADGVAGDDGHA--LSASRAAPLRSRRMIGGTLA--PLVPWQAVVYLSDEVLLGGYAGGALISDRWVLTAGRN-LFVVKSR	175
Zebrafish	TDASPALERVGEHVS-----LRSRRMIGGSLT--ASVPWQAVVYSENILLGGYAGGALISDRWVLTAGRN-LFVVKSR	172
Mouse	VAGEKLPECE-AVCGKPKHPVDQVORLIGGSDMDAGSHPWQAKMSRHGLTTC-----ATLISDQWILTARN-LFVVKSR	151
Human	AVGDKLPECE-AVCGKPKHPANPVORLIGGHLDAKGSHPWQAKMVSNNLTTC-----ATLISDQWILTARN-LFVVKSR	210
Nurse shark	DLGYEFPTCEAVCGKPIVPLEQHQRVVGGLVHNGATPVTMLGPGSTVVD-----GTLIDHHVILTSARHLEFNLRSR	234
Little skate	DLGTLPHCIPVVCGHAVTHLDSVHETDCAQLVTKHATEWALLKNAEDFHN-----GVLISHQWILTSARHLEFNLRSR	223
Gilthead seabream	QDNQCKEFLIPKVLGITQKSDNPTNEVSVEKVVLLHG--FQNSDWDNDLALILQKKAIVMSDKVPIPLPERGGQE	177
European sea bass	QDNQCKEFLIPKVLGITRSEADVSREVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	177
Rockfish	QDAGKEGFLIPKVLGITRSCANPSREVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	171
Nile tilapia	EAIQCKEFLIPKVLGITKDDANSSEVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	177
Atlantic salmon	QDTQCKEFLIPKVLGITRYSQANDSREVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	174
Rainbow trout	QDTQCKEFLIPKVLGITRYSQANDSREVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	212
Channel catfish	KDTQCKEFLIPKVLGITRYSQANDSREVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	172
Pufferfish	QDTQCKEFLIPKVLGITRYSQANDSREVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	253
Zebrafish	QDTQCKEFLIPKVLGITRYSQANDSREVAVEKVVLLHG--FQNSDWDNDLALILQKPEVMSDKVPIPLPERGGQE	210
Mouse	TASAKD-----ITPTLTYL--VGKQOLVETKVVLLHN-----HSVDDEGLIKRLQKRVSVNRPVPIPLPERGGQE	250
Human	NATAKD-----IAPTLYL--VGKQOLVETKVVLLHN-----YSQVDDEGLIKRLQKRVSVNRPVPIPLPERGGQE	250
Nurse shark	EELKEK-----LRYVVGIEDAREITAAHQVDEPVEVYHPR--MRDAYVYRNDHALQKRVSVNRPVPIPLPERGGQE	374
Little skate	EAIKID-----FVYVGVEDLDLHASHPHVERIFEEIHADNASEMDNDLVLRLSDSVSGDHVPIPLPERGGQE	298
Gilthead seabream	NTMQSGVILGWG--TFLTSATSLRLHLPLASHSCRAEYSRIP-----FTEDVDNMFCTGPA-SFERN	242
European sea bass	GIVQSGVILGWG--IYLTATSLRLHLPLASHSCRAEYERIA-----LTPSDVDNMFCTGAT-KFQGN	242
Rockfish	DTMGSGVILGWG--ILLTFSKSLRLHLPLASHSCRAEYERNP-----FTEDVDNMFCTGAT-KYDGN	236
Nile tilapia	KAVHSGVILGWG--PLLTSPFLKHLHLPLASHSCRAEYESLA-----LTETVDNMFCTGAT-KYQGN	242
Atlantic salmon	EAAQKSGVILGWG--VHFTFAESLRLHLPLASHSCRAEYNRGG-----STETVDNMFCTGAS-KYQGN	239
Rainbow trout	EAAQKSGVILGWG--VFTFAESLRLHLPLASHSCRAEYNPGGQ-----VLSSTETVDNMFCTGAS-KYQGN	281
Channel catfish	ESEQSGVILGWG--PLLTSPFLKHLHLPLASHSCRAEYERIA-----NVDGKINAILRT-EFQGN	223
Pufferfish	DGAQSGVILGWG--VYLNLSLRLHLPLASHSCRAEYEQSQ-----LAPSVDNMFCTGAT-KYQGN	319
Zebrafish	ERDQSGVILGWG--RLLTPAVLRLHLPLASHSCRAEYVLES-----TENDVDNMFCTGAS-RYQGN	314
Mouse	--FCRVGVSGWGRN--ANFRFTDRLRYVMLPVADQRCVYH--ENSTVPEKKNTLSPVGVQPIPLNEHTFCQGLT-KYQGN	290
Human	--FCRVGVSGWGRN--ANFRFTDRLRYVMLPVADQRCVYH--ENSTVPEKKNTLSPVGVQPIPLNEHTFCQGLT-KYQGN	349
Nurse shark	--EGKTGHVAGVGVEGTGETSRANHLRWVSLVAVNTLCAFFN-----EHPHGFADAPDQCTQSL-SDGN	379
Little skate	--VGVEGAVGWDLD--HAKGPHHLYVVLVPEEAKCEVHEFS-----SHHGLFEDDNLDEFCTHGL-EKQGN	362
Gilthead seabream	VCFGDAGGALAVDIPETGDIYAAGILSYDKSCRYNHGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISQOO	319
European sea bass	VCFGDAGGALAVDAKTGDIYAAGILSYDKSCRYNYGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	319
Rockfish	VCFGDAGGALAVDAETGDIYAAGILSYDKSCRYNHGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYILWQO	313
Nile tilapia	VCFGDAGGALAVDIPETGDIYAAGILSYDKSCRYNHGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	319
Atlantic salmon	VCFGDAGGALAVDIPKGRVYAAGILSYDKCAVRYGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	316
Rainbow trout	VCFGDAGGALAVDIPKGRVYAAGILSYDKCAVRYGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	358
Channel catfish	VCFGDAGGALAVLNPVTKKVYAAGILSYDPTCRNEEAVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	298
Pufferfish	VCFGDAGGALAVDIPETGDIYAAGILSYDKSCRYNHGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	396
Zebrafish	VCFGDAGGALAVLNPVTKKVYAAGILSYDKCAVRYGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	391
Mouse	TCVGDAGSABAHDMEDDTYAAGILSYDKCAVRYGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	347
Human	TCVGDAGSABAHDMEDDTYAAGILSYDKCAVRYGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	406
Nurse shark	VCFGDAGGALAVR--DGDYDYAAGILSYDKCAVRYGVYMKLSSYLFWIHTVIRGDTENSALRTDAMSKYISROP	429
Little skate	NSERDAGAVQVYS--VGHKDYAGVLAPEVKGWAVYTDVYHLDVNNVIEHN	417
Gilthead seabream	-----	57

Figure 2

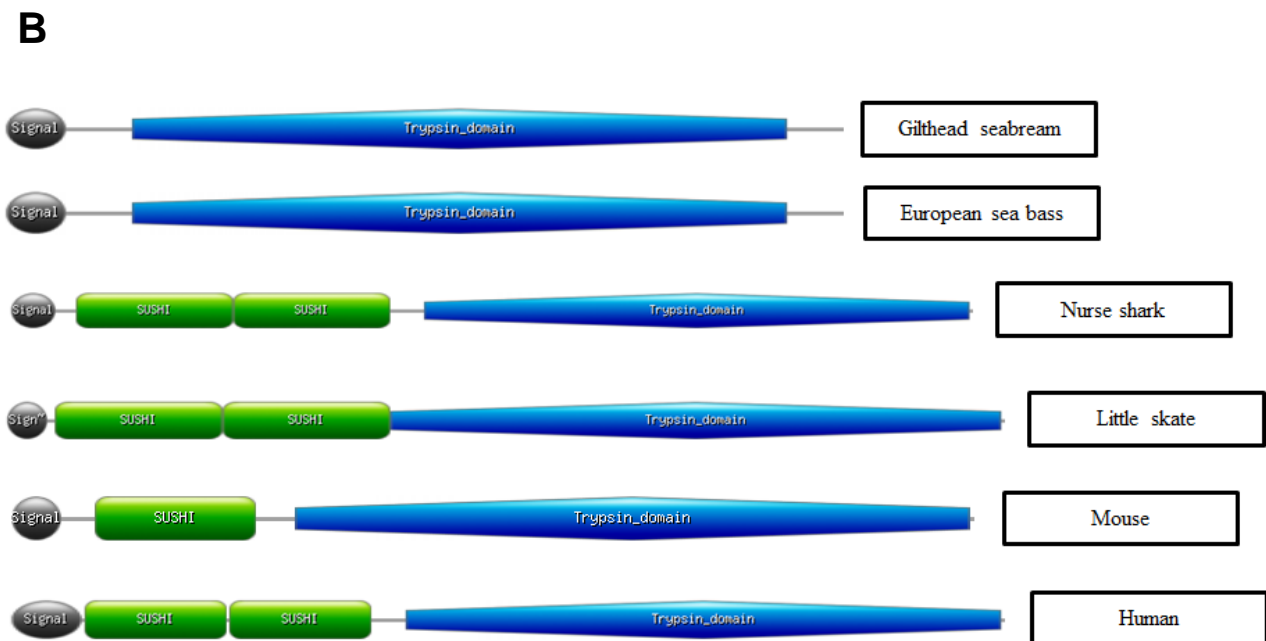
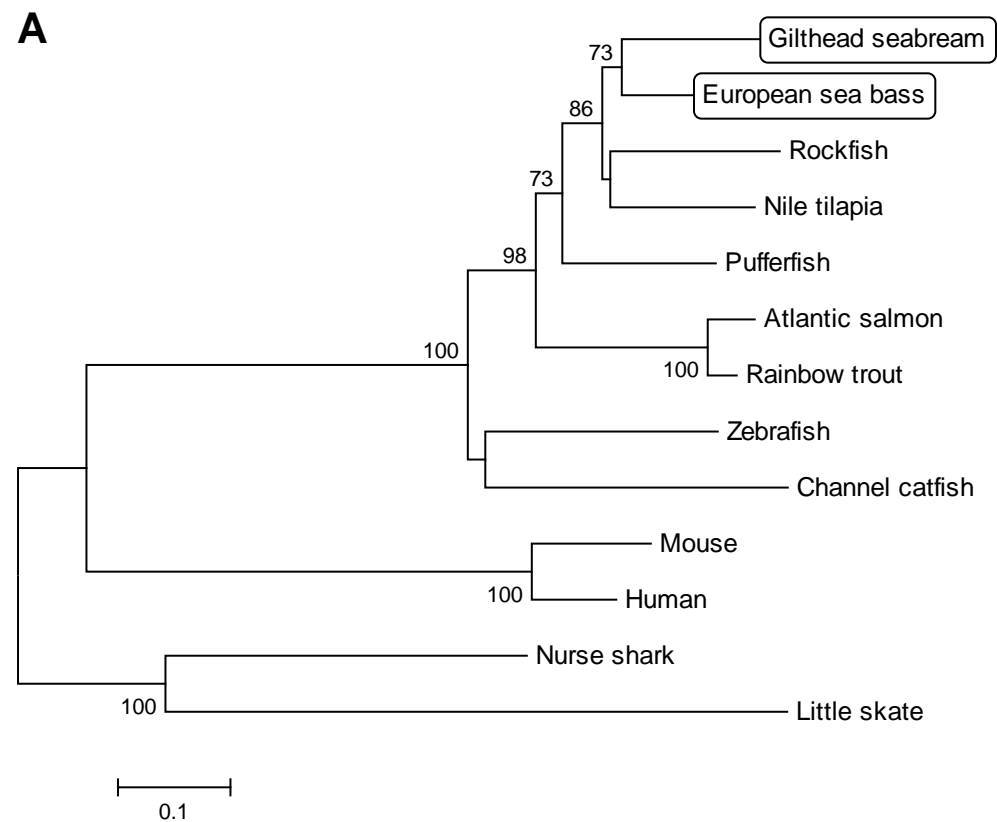


Figure 3

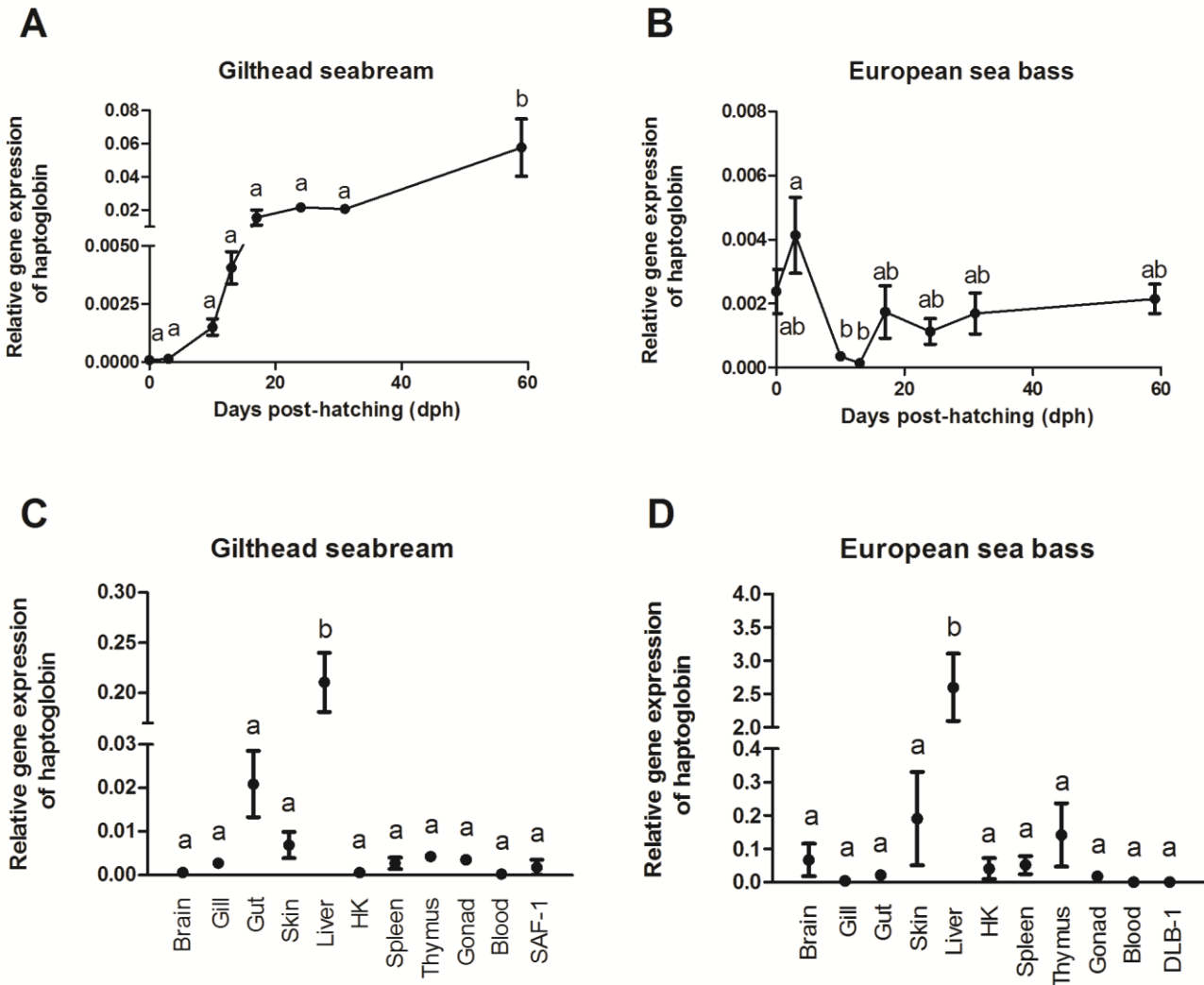


Figure 4

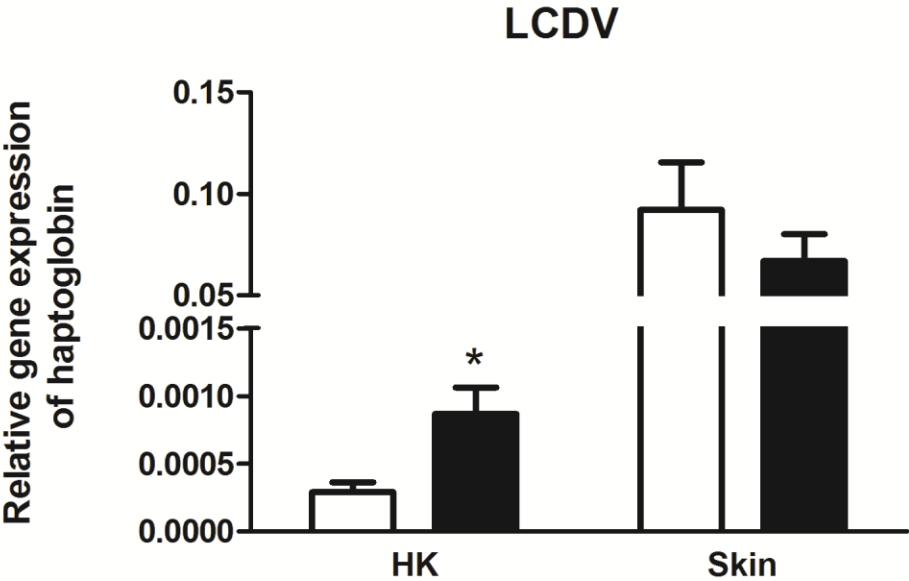
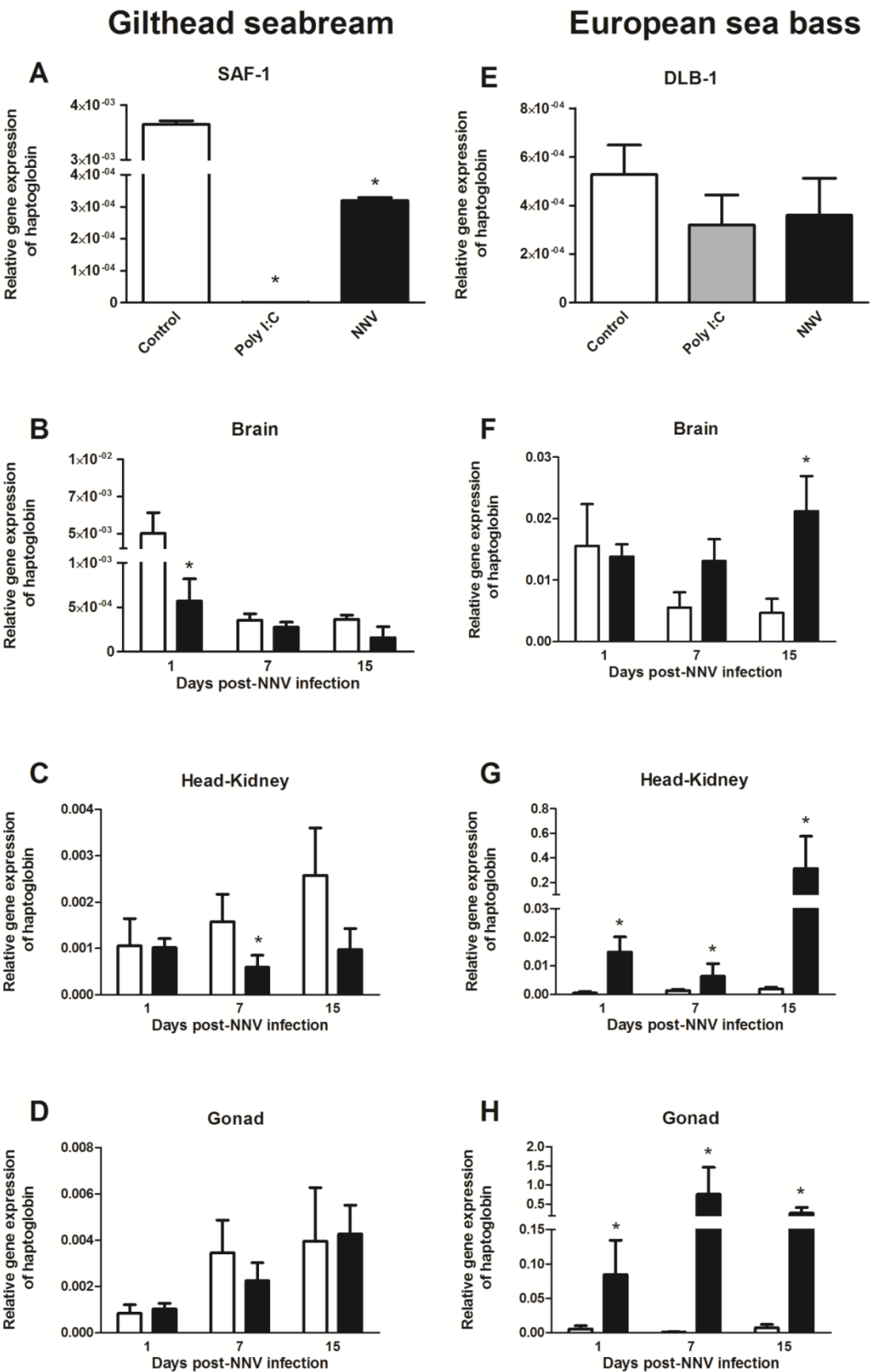


Figure 5



Supplementary Figure 1. Haptoglobin gene expression in gilthead seabream after intraperitoneal (IP) injection of PBS (white bars) or *Vibrio anguillarum* (black bars) in different tissues. . Results are expressed as the mean relative expression to the expression of endogenous control *ef1a* gene \pm SEM (n=2 pooled samples). Asterisks denote significant differences between control and infected groups when $p \leq 0.05$.

